

PA 260270

REC'D 23 AUG 2000

WIPO

PCT

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

GB00/02962

June 09, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/149,176

FILING DATE: August 17, 1999

E J U

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. Williams
N. WILLIAMS

Certifying Officer

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING PROVISIONAL PATENT APPLICATION

Under 35 USC 111(b)

(Not for DESIGN cases)

Box:

PROVISIONAL

APPLICATION

PROVISIONAL APPLICATION
Under Rule 53(c)

Commissioner of Patents
Washington, D.C. 20231

(Our Deposit Account No. 03-3975)

Herewith is a PROVISIONAL APPLICATION

Our Order No. 41301

263280

Title: IMPROVEMENTS IN OR RELATING TO RNA POLYMERASE
PROMOTERS

C#

M#

Atty. Dkt. PMS 263280

HCM/MJL/GB/C10

M#

Client Ref

including:

Date: AUGUST 17, 1999

1. Specification: 18 pages 2. ☐ Specification in non-English language 3. ☒ Drawings: 5 sheet(s)

4. The invention ☐ was ☒ was not made by, or under a contract with, an agency of the U.S. Government.

If yes, Government agency/contract # =

5. ☐ Attached is an assignment and cover sheet. Please return the recorded assignment to the undersigned.

6. ☐ Attached: (No.) Verified Statement(s) establishing "small entity" status under Rules 9 & 27.

NOTE: Do NOT File IDS!

7. ☐ Attached:

8. This application is made by the following named inventor(s) (Double check instructions for accuracy.):

(1) Inventor	Donald	L.N.	CARDY
Residence	Northamptonshire	United Kingdom	

(2) Inventor			
Residence			

(3) Inventor			
Residence			

(4) Inventor			
Residence			

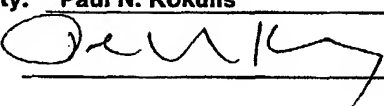
(5) Inventor			
Residence			

9. NOTE: FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet (PAT102A) with same information regarding additional inventors.

	Large/Small Entity		Fee Code
10. Filing Fee	\$150/\$75	+150	114/214
11. If "non-English" box 2 is X'd, add Rule 17(k) processing fee	\$130	+0	139
12. If "assignment" box 5 is X'd, add recording fee.	\$40	+0	581
13.	TOTAL FEE ENCLOSED =		\$150

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-17 (missing or insufficient fee only) now or hereafter relative to this application or credit any overpayment, to our Account/Order Nos. shown in the heading hereof for which purpose a duplicate copy of this sheet is attached.

Pillsbury Madison & Sutro LLP
Intellectual Property Group
 1100 New York Avenue, N.W.
 Ninth Floor East Tower
 Washington, D.C. 20005-3918
 Tel: (202) 861-3000
 Atty/Sec: PNK/sdm

By: Atty: Paul N. Kokulis
 Sig: 
 Reg. No. 16773
 Fax: (202) 822-0944
 Tel: (202) 861-3503

NOTE: File in duplicate with 2 post card receipts (PAT-103) & attachments

554780-9476479

APPLICATION UNDER UNITED STATES PATENT LAWS

Invention: IMPROVEMENTS IN OR RELATING TO RNA POLYMERASE
PROMOTERS

Inventor(s): CARDY, Donald Leonard Nicholas

Pillsbury Madison & Sutro LLP
Intellectual Property Group
1100 New York Avenue, N.W.
Ninth Floor, East Tower
Washington, D.C. 20005-3918
Attorneys
Telephone: (202) 861-3000

This is a:

- ☒ Provisional Application
- ☐ Regular Utility Application
- ☐ Continuing Application
- ☐ PCT National Phase Application
- ☐ Design Application
- ☐ Reissue Application
- ☐ Plant Application
- ☐ Substitute Specification
Sub. Spec. filed _____
in App. No. _____ / _____
- ☐ Marked Up Specification re
Sub. Spec. filed _____
in App. No. _____ / _____

SPECIFICATION

C1092.01/C

Title: Improvements in or Relating to RNA Polymerase Promoters**Field of the Invention**

This invention relates to nucleic acid complexes comprising a functional RNA polymerase promoter, and to a method of detecting a target nucleic acid sequence of interest.

Background of the Invention

RNA polymerases are enzyme molecules well-known to those skilled in the fields of molecular biology and molecular diagnostic kits. RNA polymerases synthesise RNA molecules from a DNA template strand.

Much research has been carried out on RNA polymerases, especially bacteriophage RNA polymerases. Generally, bacteriophage RNA polymerases are exceptionally active for *in vitro* transcription. This high level activity may be due in part to the fact that they are composed of a single polypeptide chain and do not require a dissociating initiation factor. These polymerases have been shown to be more active on supercoiled templates although they are also very active on linear templates (Smeeckens & Romano 1986 Nucl. Acids Res. 14, 2811).

Specifically, the RNA polymerase from the bacteriophage T7 has been shown to be very selective for specific promoters that are rarely encountered in DNA unrelated to T7 DNA (Chamberlin *et al.* 1970 Nature 228, 227; Dunn & Studier 1983 J. Mol. Biol. 166, 477). T7 RNA polymerase is able to make complete transcripts of almost any DNA that is placed under control of a T7 promoter. T7 RNA polymerase is a highly active enzyme that transcribes about five times faster than does *Escherichia coli* RNA polymerase (Studier *et al.* 1990 Methods Enzymol. 185, 60). The synthesis of small RNAs using T7 RNA polymerase has been described whereby sequences around the RNA polymerase promoter sequence are shown to be important in the reproducible improvement of yield of RNA produced (Milligan & Uhlenbeck, 1989 Methods Enzymol. 180, 51 and Milligan *et al.* 1987 Nucl. Acids Res. 15, 8783-8798). Other RNA polymerases that have similar

654750-926109

properties to T7 include those from bacteriophage T3 and SP6, the genes for which have all been cloned and the corresponding enzymes are commercially available. The optimum promoter sequences for T7, T3 and SP6 polymerases are known, and are essentially 17 nucleotides long.

A number of methods have been disclosed, which utilise RNA polymerases to synthesise RNA directly or indirectly as the result of the presence of a particular nucleic acid sequence of interest ("target"). The presence of RNA (detected directly or indirectly) thus serves to signal the presence of the sequence of interest and can be used as the basis of assay methods and/or diagnostic methods or kits. Examples include the disclosures of WO 93/06240, WO 94/29481, EP 0851033, and EP 0552931.

Summary of the Invention

In a first aspect the invention provides a nucleic acid complex formed by a hybridisation reaction comprising four nucleic acid molecules; the complex comprising a target nucleic acid molecule and first, second and third nucleic acid probe molecules; wherein the first probe comprises a foot region which is complementary to a first portion of the target and is hybridised thereto, and an arm region which is substantially non-complementary to the target; the second probe comprises a foot region which is complementary to a second portion of the target, such that the foot region of the second probe is hybridised to the target adjacent or substantially adjacent to the foot region of the first probe, the second probe also comprising an arm region which is substantially non-complementary to the target but which is complementary and hybridised to the arm region of the first probe; the third probe being complementary, at least in part, to a portion of the arm region of the first probe, such that third probe is hybridised to the arm region of the first probe adjacent to the second probe; and wherein formation of the complex creates a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, and the other strand being provided jointly by the second probe and by the third probe.

Preferably the sequence of the probes and the hybridisation reaction conditions are selected such that the first, second and third probes cannot become hybridised in the absence of the

50149175-081799

target, such that formation of the RNA polymerase promoter occurs in a target-dependent manner.

In a second aspect, the invention provides a method of detecting the presence of a target nucleic acid molecule in a sample, the method comprising the steps of contacting the sample comprising the target with first and second nucleic acid probes, each probe comprising a foot region complementary to respective first and second portions of the target, which portions are adjacent or substantially so; wherein the first and second probes each further comprise an arm region substantially non-complementary to the target, at least part of the arm region of the first probe being complementary to at least part of the arm region of the second probe, such that respective foot regions of the first and second probes hybridise to the target, allowing hybridisation of the complementary parts of the arm regions of the first and second probes; and causing to be present a third nucleic acid probe molecule which is complementary to a portion of the arm region of the first probe, such that the third probe hybridises to the first probe adjacent to the arm region of the second probe, thereby creating a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, the other strand being provided jointly by the second and third probes; causing RNA synthesis from the RNA promoter; and detecting, directly or indirectly, the RNA so synthesised.

The method of the second aspect of the invention thus results in formation of the complex of the first aspect.

It is an essential feature of the invention that the first and second probes, when hybridised to the target sequence, are adjacent or substantially adjacent to each other. Use of the term "adjacent" is herein intended to mean that there are no nucleotides of the target sequence left without base-pairing between those portions of the target sequence which are base-paired to the complementary sequence of the probes. This proximity between the probes enables the complementary arm portions of the probes to anneal. As will readily be apparent to those skilled in the art, by designing the probes so as to allow for annealing to each other at greater separations from the target sequence, gaps may be introduced between the foot in the target nucleotide sequence to which the probes hybridise. In this

situation the probes are said to be "substantially adjacent", because there may be some nucleotides of the target sequence left without base-pairing between those portions of the target sequence which are base-paired to the probes. Clearly, the number of intervening un-paired nucleotides of the target sequence can vary according to the design of the probes. Thus whilst it is preferred that the first and second probes hybridise so as to be adjacent, the probes may be separated by up to 5 nucleotides of target sequence, and the term "substantially adjacent" is intended to refer to such situations.

It will be apparent to those skilled in the art that the order of addition of probes in the formation of the nucleic acid complex is not critical: the third probe may, for example, be hybridised to the first probe before the second probe and sample are added. Alternatively, for example, all three probes may be simultaneously mixed with the sample containing the target molecule.

It will be further apparent to those skilled in the art that the second and third probes, which jointly provide one of the strands of the RNA polymerase promoter, are not covalently joined and the promoter sequence thus contains a "nick" in the phosphodiester backbone of one of the strands. As far as the inventors are aware, it has not been appreciated hitherto that such discontinuous nucleic acid strands could still give rise to a functional RNA polymerase promoter.

Preferred promoters for use in the invention are those recognised by bacteriophage polymerases, especially those promoters recognised by one of T3, T7 or SP6 polymerase. These will generally comprise a minimum of 17 bases, essentially double-stranded. The sequence of the double-stranded T3 RNA polymerase promoter (described in the prior art) is:

5' AAATTAACCCTCACTAAA 3'
3' TTAAATTGGGAGTGATTT 5'

(A number of variant T3 promoter sequences are also known, especially those in which the first three bases of the non-template strand [the upper strand shown above] are 5' TTA

50149176.001799

3', rather than AAA.)

The sequence of the T7 RNA polymerase promoter (described in the prior art) is:

5' TAATACGACTCACTATA 3'
3' ATTAAGCTGAGTGATAT 5'

The sequence of the SP6 RNA polymerase promoter (described in the prior art) is:

5' ATTTAGGTGACACTATA 3'
3' TAAATCCACTGTGATAT 5'

One of the strands is provided by the first probe. Typically this will be the "sense" (+) strand, which is transcribed by the polymerase. Accordingly the first probe will generally comprise a stretch of "template" nucleic acid to be transcribed. The template will desirably comprise sequences which facilitate capture (e.g. hybridisation) of the resulting RNA transcript and/or detection. In addition, the first probe may desirably contain sequences (e.g. a "+12 sequence") 5' of the promoter sequence, which serve to increase the activity of the promoter. Specific instances of such sequences are disclosed in the examples below.

The inventors have elucidated the optimum sequence of +12 regions for the T7 polymerase (discussed in greater detail below) - it is not known at present if these are also optimum for, say, T3 and SP6 polymerases. If, as is possible, SP6 and T3 polymerases have different optimum +12 regions, it would be a simple matter for the person skilled in the art to identify the relevant sequence by trial-and-error, with the benefit of the present disclosure.

The sequences of preferred +12 regions, for inclusion in the template portion of the first probe, (in respect of T7 polymerase) are shown below in Table 1. The most active +12 region (giving greatest transcription) is at the top, with the other sequences shown in decreasing order of preference.

Table 1 Alternative template +1 to +12 sequences for T7 polymerase, in descending order of transcription efficiency.

5' ATCGTCAGTCCC 3'
5' GCTCTCTCTCCC 3'
5' ATCCTCTCTCCC 3'
5' GTTCTCTCTCCC 3'
5' GATGTGTCTCCC 3'
5' GTTGTGTCTCCC 3'
5' ATCCTCGTGCCC 3'
5' GCTCTCGTGCCC 3'
5' GTTCTCGTGCCC 3'
5' GTTGTGCGTGCCC 3'

(The 5' base is numbered as +1, being the first base downstream from the end of the promoter sequence, the 3' base as +12).

In a further embodiment, the template portion of the complex (on the first probe) could contain sequences that can be used to identify, detect or amplify the *de novo* synthesised RNA transcripts (see, for example, WO 93/06240, US 5,554,516, or, for example, using molecular beacon sequences such as those disclosed by Tyagi & Kramer 1996 Nature Biotech 14, 303-308). These sequences are conveniently placed adjacent to, and downstream of, a +12 region (as described above) and may comprise, but are not limited to, one or more of the following: unique "molecular beacon" sequences; capture sequences; and detection probe complementary sequences.

In principle, the second and third probes may make any contribution to the promoter sequence provided that, in combination, the second and third probes provide one strand of the full promoter sequence. In practice, the inventors have found that optimum results are generally obtained when the second probe provides 2 to 4 (preferably 3) bases at the 5' end of the promoter sequence, with the rest of the promoter (15 to 13 bases) being contributed by the third probe.

50149176.081799

Any of the first, second or third probes may comprise DNA, peptide nucleic acid (PNA), locked nucleic acid (LNA), (less preferably RNA) or any combination thereof. It will, however, generally be desirable that those portions of the probes which constitute the promoter comprise conventional DNA, so as to ensure recognition by the relevant polymerase.

PNA is a synthetic nucleic acid analogue in which the sugar/phosphate backbone is replaced by a peptide-linked chain (typically of repeated N-(2-aminoethyl)-glycine units), to which the bases are joined by methylene carbonyl linkages. PNA/DNA hybrids have high Tm values compared to double stranded DNA molecules, since in DNA the highly negatively-charged phosphate backbone causes electrostatic repulsion between the respective strands, whilst the backbone of PNA is uncharged. Another characteristic of PNA is that a single base mis-match is, relatively speaking, more destabilizing than a single base mis-match in heteroduplex DNA. Accordingly, PNA is useful to include in probes for use in the present invention, as the resulting probes have greater specificity than probes consisting entirely of DNA. Synthesis and uses of PNA have been disclosed by, for example, Orum *et al.*, (1993 Nucl. Acids Res. 21, 5332); Egholm *et al.*, (1992 J. Am. Chem. Soc. 114, 1895); and Egholm *et al.*, (1993 Nature 365, 566).

LNA is a synthetic nucleic acid analogue, incorporating "internally bridged" nucleoside analogues. Synthesis of LNA, and properties thereof, have been described by a number of authors: Nielsen *et al.*, (1997 J. Chem. Soc. Perkin Trans. 1, 3423); Koshkin *et al.*, (1998 Tetrahedron Letters 39, 4381); Singh & Wengel (1998 Chem. Commun. 1247); and Singh *et al.*, (1998 Chem. Commun. 455). As with PNA, LNA exhibits greater thermal stability when paired with DNA, than do conventional DNA/DNA heteroduplexes. However, LNA can be synthesised on conventional nucleic acid synthesising machines, whereas PNA cannot. Therefore, in some respects, LNA is to be preferred over PNA, for use in probes in accordance with the present invention.

In addition to non-conventional nucleic acids (such as LNA, PNA or nucleic acids containing base analogues), any one or more of the probes of use in the invention may comprise one or more destabilizing moieties.

50149176-001799

The destabilizing moiety is a chemical entity which is generally unable to undergo base pairing and hydrogen bonding in the normal manner as usually occurs when complementary strands of nucleic acid become hybridised.

All manner of molecules may be suitable for use as a destabilizing moiety, although some compounds are specifically preferred, as described below. With the benefit of the present specification, the person skilled in the art will be able to test other compounds and readily select those which confer the appropriate degree of destabilization so as to prevent the hybridization of probes in the absence of target nucleic acid of interest. Particularly preferred, as a matter of convenience, are those compounds which are commercially available in a form (e.g. as phosphoramidites) which facilitates their incorporation into synthetic oligonucleotides using conventional automated solid phase nucleic acid synthesisers.

Destabilizing moieties which cannot base pair, but which nevertheless are capable of forming flexible folds and/or hairpin structures, are especially suitable. One such preferred destabilizing moiety comprises hexaethylene glycol (abbreviated herein as "Hex") (see Figure 5), which may be present singly or in tandem up to n times (where n can be any number ≥ 1 , but conveniently has a maximum value of 5). In a particularly preferred embodiment, the third probe comprises one Hex molecule, where the number of bases opposite the destabilising moiety in the arm region of the first probe should be three to four bases. An alternative preferred destabilizing moiety comprises a plurality of alkylene (especially methylene) repeats. Particularly preferred are penta- or hexamethylene spacers.

Other, less preferred, destabilizing moieties may alternatively be used. These include, but are not limited to, inosine, VirazoleTM (N[1]-[1- β -D-ribofuranosyl]-3 carboxamido-1,2,4-triazole), NebularinTM (N[9]-[1- β -D-ribofuranosyl]-purine), nitroproline, ribose, propyl or combinations of the above eg. propyl-Hex-propyl, propyl-Hex-Hex-propyl, etc. Propyl may be replaced by, for example, ethyl, butyl, pentyl, heptyl, octyl etc. The number of bases opposite the destabilizing moiety in the arm region of the reciprocal probe should be x , where x is ≥ 1 . The exact number of bases will of course depend on the size of the

destabilizing moiety and the value of n .

The following may be used as a guide. for each Hex molecule in the destabilizing moiety, the opposite probe should preferably comprise 3-4 bases (preferably 3); for each other molecule or radical mentioned above present in the destabilizing moiety, the opposite probe should preferably comprise a single base, with the exception of the following: butyl - two bases, pentyl - two bases, heptyl - three bases, and octyl - four bases.

The chemicals described above and used as destabilizing moieties are all commercially available (e.g. from Glen Research, USA).

The person skilled in the art will appreciate how to select appropriate conditions, materials and sequences for the probes, in order to ensure that the complex of the first, second and third probes (and hence formation of the functional RNA promoter) occurs in a target dependent manner. In essence, the degree of complementarity between the arm regions of the first and second probes must be such that, in the conditions employed, they will not hybridise unless stabilised by hybridisation of the respective foot regions of the first and second probes to the target.

Generally therefore, the foot regions of the first and second probes will comprise at least 10 bases, preferably at least 20 bases, and more preferably at least 25 bases. There is no upper limit on the size of the foot regions (which may, for example, comprise several kilobases). However, in practice, the probes will normally be *in vitro* synthesised oligonucleotides and so it will be preferred for the foot regions to comprise no more than about 75 bases.

In contrast, the number of complementary bases between the arm regions of the first and second probes will normally be no more than 25, typically less than 15, and optimally between 5 and 13 bases.

In preferred embodiments, the invention provides a method of generating a signal in a target-dependent manner (i.e. creation of the complex and hence formation of the

10

functional promoter) and causing amplification of this signal (generation of multiple RNA transcripts under the control of the promoter) in a system which may require the use of a single enzyme type (RNA polymerase), without the need for additional enzymes (e.g. DNA polymerases) to bring about the amplification. This is significant as the reaction conditions for optimum activity of RNA and DNA polymerases are generally mutually exclusive.

Detection Methods

RNA produced in accordance with the invention could be detected in a number of ways, preferably following amplification (most preferably by means of an isothermal amplification step e.g. as disclosed in US 5,399,491 and US 5,480,784). For example, newly-synthesised RNA could be detected in a conventional manner (e.g. by gel electrophoresis), with or without incorporation of labelled bases during the synthesis.

Alternatively, for example, newly-synthesised RNA could be captured at a solid surface (e.g. on a bead, or in a microtitre plate), and the captured molecule detected by hybridisation with a labelled nucleic acid probe (e.g. radio-labelled, or more preferably labelled with an enzyme, chromophore, fluorophore and the like). Preferred enzyme labels include horseradish peroxidase and alkaline phosphatase.

One preferred detection method involves the use of molecular beacons or the techniques of fluorescence resonance energy transfer ("FRET"), delayed fluorescence energy transfer ("DEFRET") or homogeneous time-resolved fluorescence ("HTRF"). Molecular beacons are molecules which a fluorescence signal may or may not be generated, depending on the conformation of the molecule. Typically, one part of the molecule will comprise a fluorophore, and another part of the molecule will comprise a "quencher" to quench fluorescence from the fluorophore. Thus, when the conformation of the molecule is such that the fluorophore and quencher are in close proximity, the molecular beacon does not fluoresce, but when the fluorophore and the quencher are relatively widely-separated, the molecule does fluoresce. The molecular beacon conveniently comprises a nucleic acid molecule labelled with an appropriate fluorophore and quencher.

50149175-081799

One manner in which the conformation of the molecular beacon can be altered is by hybridisation to a nucleic acid, for example inducing looping out of parts of the molecular beacon. Alternatively, the molecular beacon may initially be in a hair-pin type structure (stabilised by self-complementary base-pairing), which structure is altered by hybridisation, or by cleavage by an enzyme or ribozyme.

FRET (Fluorescence Resonance Energy Transfer) occurs when a fluorescent donor molecule transfers energy via a nonradiative dipole-dipole interaction to an acceptor molecule. Upon energy transfer, which depends on the R^{-6} distance between the donor and acceptor, the donor's lifetime and quantum yield are reduced and the acceptor fluorescence is increased or sensitised.

Another approach (DEFRET, Delayed Fluorescence Energy Transfer) has been to exploit the unique properties of certain metal ions (Lanthanides e.g. Europium) that can exhibit efficient long lived emission when raised to their excited states ($\lambda_{\text{excitation}} = 337 \text{ nm}$, $\lambda_{\text{emission}} = 620 \text{ nm}$). The advantage of such long lived emission is the ability to use time resolved (TR) techniques in which measurement of the emission is started after an initial pause, so allowing all the background fluorescence and light scattering to dissipate. Cy5 (Amersham Pharmacia) ($\lambda_{\text{excitation}} = 620 \text{ nm}$, $\lambda_{\text{emission}} = 665 \text{ nm}$) can be used as the DEFRET partner.

HTRF (see WO92/01224; US 5,534,622) occurs where the donor (Europium) is encapsulated in a protective cage (cryptate) and attached to the 5' end of an oligomer. The acceptor molecule that has been developed for this system is a protein fluorophore, called XL665. This molecule is linked to the 3' end of a second probe. This system has been developed by Packard.

In another embodiment, the newly-synthesised RNA, before or after amplification, results in formation of a ribozyme, which can be detected by cleavage of a particular nucleic acid substrate sequence (e.g. cleavage of a fluorophore/quencher dual-labelled oligonucleotide).

The invention will now be further described by way of illustrative example and with

12

reference to the accompanying drawings, wherein:

Figures 1 and 3 are schematic representations of a nucleic acid complex in accordance with the invention;

Figures 2 and 4 are bar charts showing amount of RNA produced (in femtomoles) from various nucleic acid complexes, and;

Figure 5 is a schematic representation of a preferred destabilizing moiety for use in some embodiments of the invention.

Example 1

This example demonstrated that T7 RNA polymerase and three oligonucleotide probes could be used to detect a specific target sequence. Probe 1 contained a 34 base foot region complementary to the target sequence; an 8 base overlap sequence; the 17 base template strand of the T7 promoter; a +12 sequence and a capture and probe sequence for detection. Probe 2 comprised a 31 base foot region complementary to the target sequence; an 8 base overlap sequence and the first three bases of the complementary strand of the T7 promoter. Probe 3 contained the remaining 14 bases of the complementary strand of the 17 base T7 promoter sequence. The example is illustrated schematically in Figure 1.

1.1 Preparation of oligonucleotides

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturer's proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

1.2 Synthesis of RNA off hybridised oligonucleotides

Hybridisation was achieved in an assay mixture that contained 10 fmol probe 1, 50 fmol

60149175-001799

of probe 2, 25 fmol of probe 3 and 1 fmol of probe 4 (target for CFTR gene), together with T7 RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl at final concentration). The reaction volume was made up to 20 µl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3 without target (probe 4). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids, then cooled (by ramping at 0.1°C/second) to 37°C. T7 RNA polymerase (25 units) and 2 µl NTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)) were added and the mixture incubated at 37°C for 3 hours.

1.3 Capture and detection of synthesised RNA

20 µl of assay sample was added to 145 µl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (specific for the RNA to be detected) and 6 pmol specific alkaline phosphatase oligonucleotide in streptavidin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figure 2.

1.4 List of oligonucleotides

Probe 1

5' TGCCTCCTTGTCTCCGTTCTGGATATCACCGATGTGGCTCTCTCTCCCTA
TAGTGAGTCGTATTAATTTTGAAGGTGTTTCCTATGATGAATATAGATACAGA
AGCG 3' (phosphate blocked)

Probe 2

5' GCCTGGCACCATTAAAGAAAATATCATCTTTTCGAAATTAA 3'

Probe 3

14

5' TACGACTCACTATA 3'

Probe 4 (target)

5' GTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATAGGAAACAC
CAAAGATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGGAAAACCTGAGA
ACAGAAAGAAATCTTC 3'

Sequence of transcribed RNA

5' GGGAGAGAGAGCCACAUCGGGUGAUAUCCAGAACGGAGACAAGGAG
GCA 3'

Capture Probe

5' TCTGCTGCCTGCTTGTCTGCGTTCT 3'

Detection probe (Wallac-AP)

5' GGATATCACCCG 3' (3' alkaline phosphatase labelled)

Example 2

This example demonstrated that a Hexaethylene glycol linker (Hex) positioned 3 bases from the 3' end of Probe 3 increased the amount of signal obtained. The example is illustrated schematically in Figure 3.

2.1 Preparation of oligonucleotides

All oligonucleotide probes were prepared and functionalised as described in Example 1.1. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxyuridylyl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. All oligonucleotides were HPLC purified using standard techniques.

2.2 Synthesis of RNA off hybridised oligonucleotides

Hybridisation was achieved in an assay mixture that contained 10 fmol probe 1, 50 fmol of probe 2, 50 fmol of probe 3 or probe 5 and 1 fmol of probe 4 (target for CFTR gene), together with T7 RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl at final concentration). The reaction volume was made up to 20 µl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3 without target (probe 4). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids, then cooled (by

ramping at 0.1°C / second) to 37°C. T7 RNA polymerase (25 units) and 2 µl NTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)) were added and the mixture incubated at 37°C for 3 hours.

2.3 Capture and detection of synthesised RNA

20 µl of assay sample was processed as described in Example 1.3. Results are presented in Figure 4.

2.4 List of oligonucleotides

Probe 1

5' TGCCCTCTTGTCTCCGTTCTGGATATCACCCGATGIGGCTCTCTCTCCCTA
TAGTGAGTCGTATTAAATTCGAAGGTGTTTCTCTATGATGAATATAGATACAGA
AGCG 3' (phosphate blocked)

Probe 2

5' GCCTGGCACCATTAAAGAAAATATCATCTTTTTCGAAATTAA 3'

Probe 3

5' TACGACTCACTATA 3'

Probe 4 (target)

5' GTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATAGGAAACAC
CAAAGATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGGAAAACCTGAGA
ACAGAAATGAAATTCTTC 3'

Probe 5 H = Hexaethylene glycol

5' TACGACTCACTHATA 3'

Sequence of transcribed RNA

5' OGGACAGAGAGCCACAUCGGGUGAUAUCCAGAACGGAGACAAGGAG
GCA 3'

16-AUG-89 MON 17:06

TH W NASH & Co.

FAX NO. 0122 24353

P. 17

16

Capture Probe

5' TCTGCTGCCTGCTTGTCTGCGTTCT 3'

Detection probe (Wallac-AP)

5' GGATA TCACCCG 3' (3' alkaline phosphatase labelled)

664780-9276109

Claims

1. A nucleic acid complex formed by a hybridisation reaction comprising four nucleic acid molecules; the complex comprising a target nucleic acid molecule and first, second and third nucleic acid probe molecules; wherein the first probe comprises a foot region which is complementary to a first portion of the target and is hybridised thereto, and an arm region which is substantially non-complementary to the target; the second probe comprises a foot region which is complementary to a second portion of the target, such that the foot region of the second probe is hybridised to the target adjacent or substantially adjacent to the foot region of the first probe, the second probe also comprising an arm region which is substantially non-complementary to the target but which is complementary and hybridised to the arm region of the first probe; the third probe being complementary, at least in part, to a portion of the arm region of the first probe, such that third probe is hybridised to the arm region of the first probe adjacent to the second probe; and wherein formation of the complex creates a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, and the other strand being provided jointly by the second probe and by the third probe.

2. A method of detecting the presence of a target nucleic acid molecule in a sample, the method comprising the steps of contacting the sample comprising the target with first and second nucleic acid probes, each probe comprising a foot region complementary to respective first and second portions of the target, which portions are adjacent or substantially so; wherein the first and second probes each further comprise an arm region substantially non-complementary to the target, at least part of the arm region of the first probe being complementary to at least part of the arm region of the second probe, such that respective foot regions of the first and second probes hybridise to the target, allowing hybridisation of the complementary parts of the arm regions of the first and second probes; and causing to be present a third nucleic acid probe molecule which is complementary to a portion of the arm region of the first probe, such that the third probe hybridises to the first probe adjacent to the arm region of the second probe, thereby creating a functional double-stranded RNA polymerase promoter, one strand of the

18

promoter being provided by the first probe, the other strand being provided jointly by the second and third probes; causing RNA synthesis from the RNA promoter; and detecting, directly or indirectly, the RNA so synthesised.

664750-92164709

Fig. 1

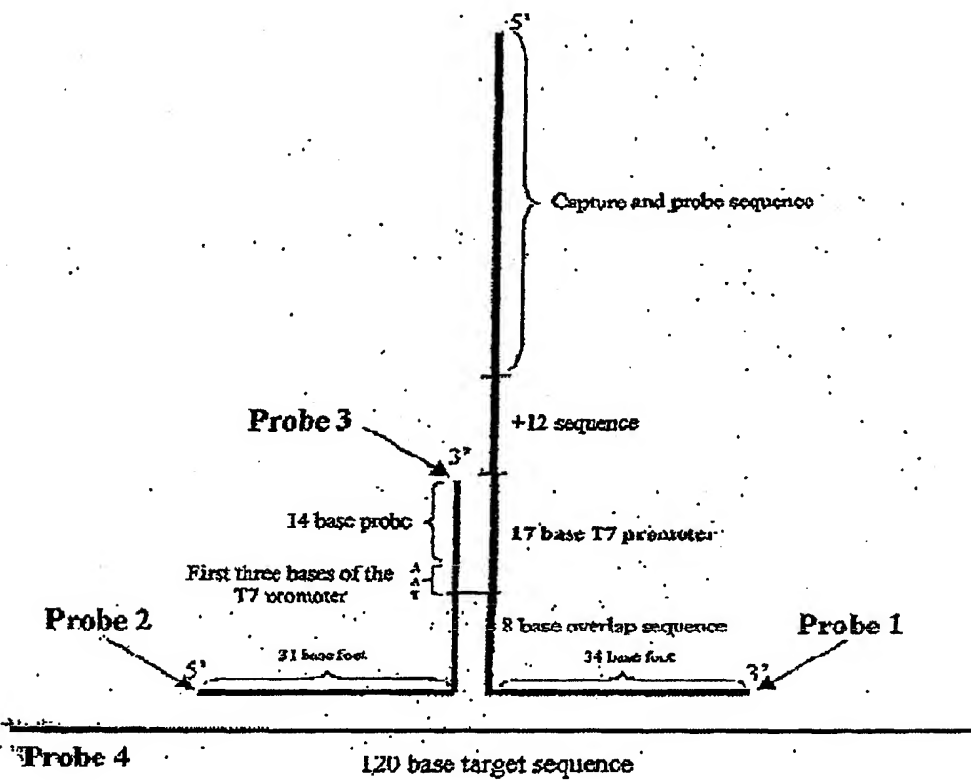
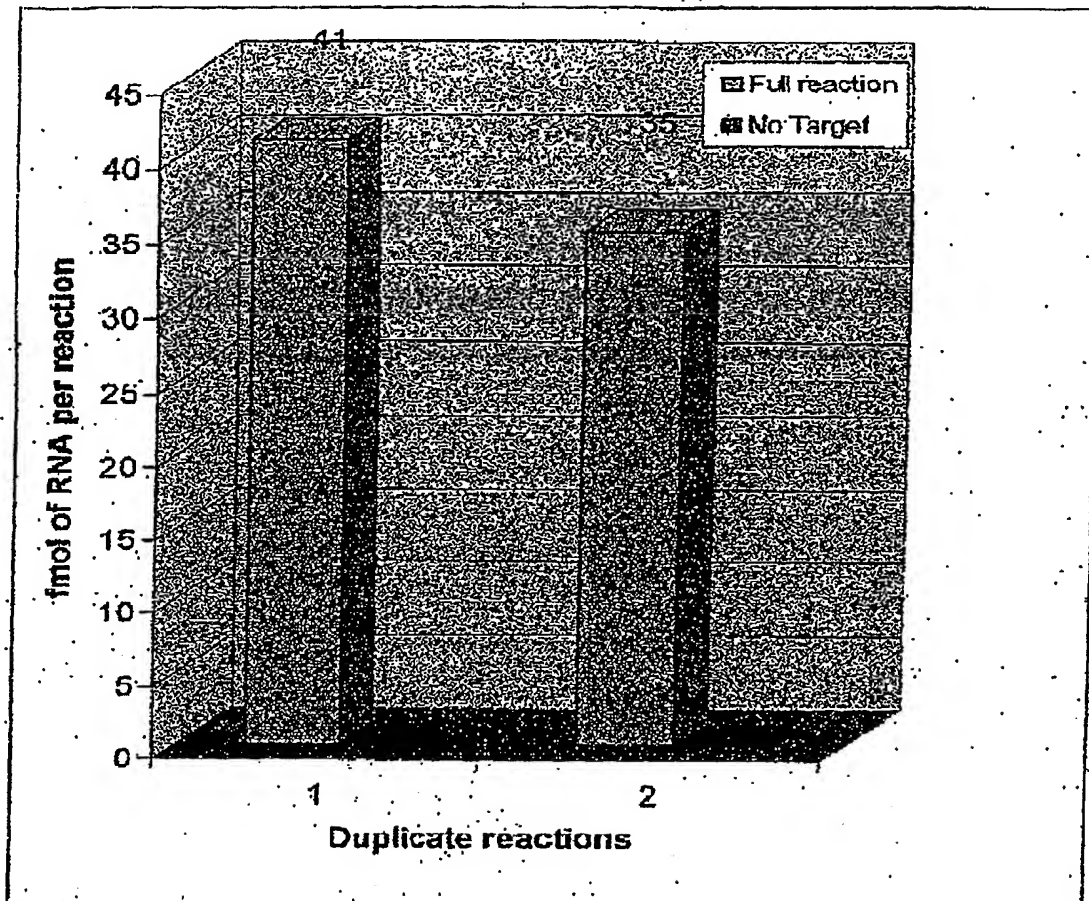


Figure 2: Detection of 1 fmol target.



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

652180-9276409

Fig. 3

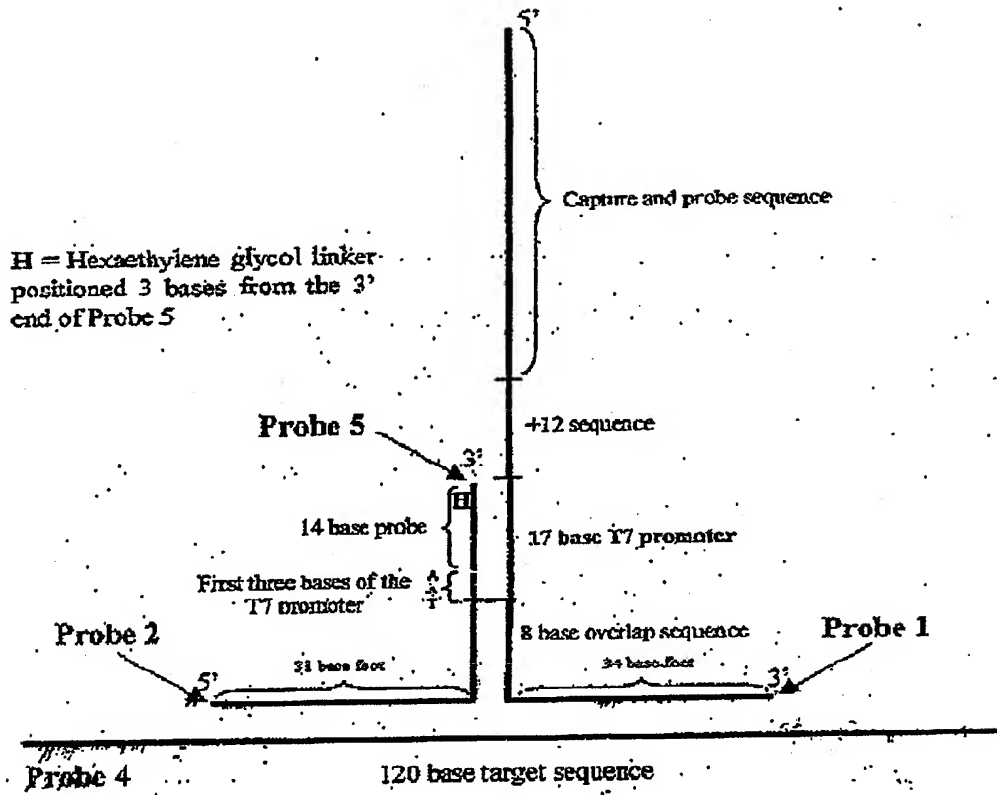
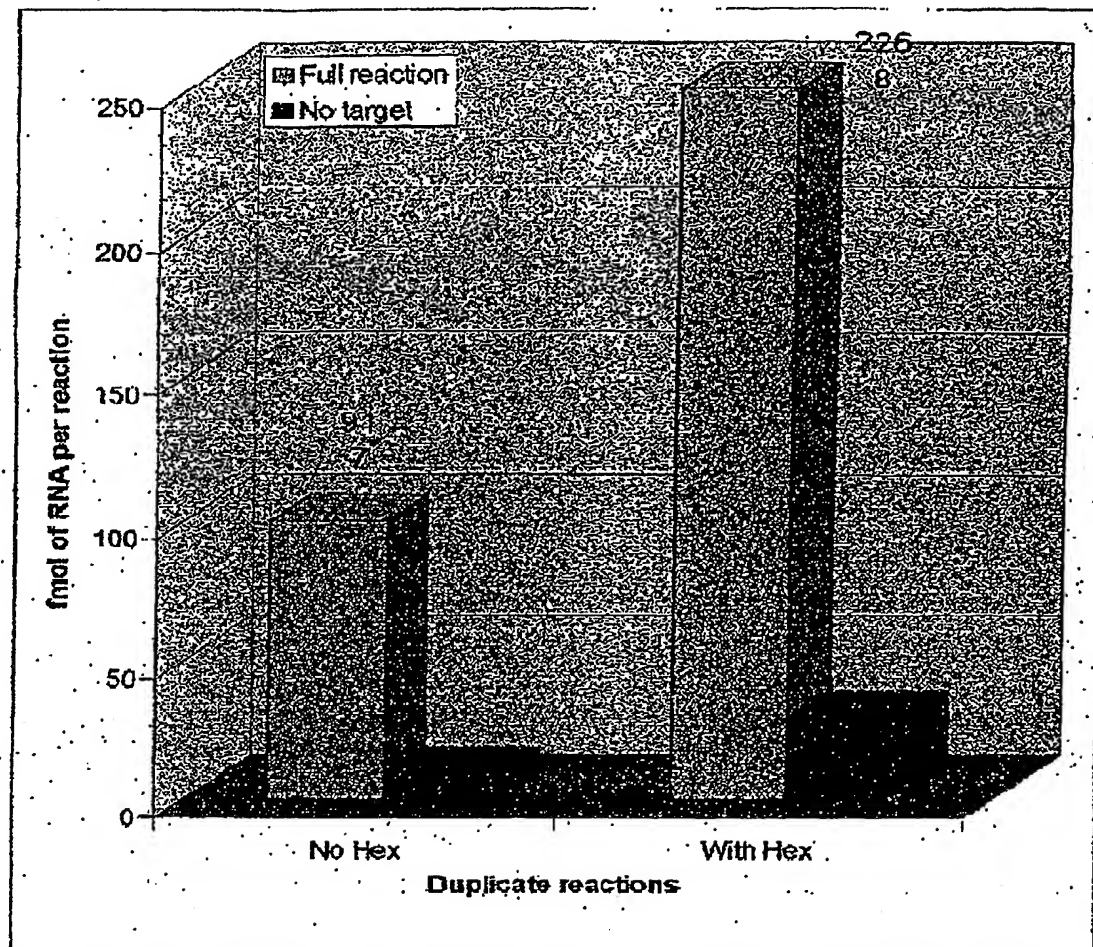


Figure 4. Effect of Hex linker in the 14 base probe.



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

